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(54) Title: CONDITIONALLY IMMORTALIZED GERM CELL LINES

(57) Abstract

The invention relates to immortalized and conditionally immortalized germ cell lines, and in particular to immortalized and conditionally immortalized testicular cell lines. Seminiferous-tubule-like structures can be produced *in vitro* using the immortalized cell lines of the present invention. Methods of producing such cell lines are also provided as well as methods for the *in vitro* production of proteins expressed by these cell lines. In a further aspect, the present invention provides methods of controlling the proliferation and differentiation of immortalized germ cells for a variety of purposes, including *in vitro* fertilization and the production of transgenic mice.

CONDITIONALLY IMMORTALIZED GERM CELL LINES

This invention was made with government support under research grant CA-42595 awarded by the National Institutes of Health and grant number CA30199, a Cancer Center Support grant. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The present invention generally relates to immortalized and conditionally immortalized cell lines, each associated with spermatogenesis, and uses for such cell lines.

Mature sperm cells in animals originate from germ cells that have undergone a process referred to as spermatogenesis. This process takes place within a specialized microenvironment of the seminiferous or sperm-producing tubule located in the testis.

In maturing to sperm cells, germ cells progress through several stages of differentiation during this process. For example, mouse germ cells proceed through twelve stages of meiotic differentiation, including primitive type A, type A and type B spermatogonia, preleptotene, leptotene, zygotene and pachytene spermatocytes (generally referred to as primary spermatocytes), secondary spermatocytes and round spermatids. Further differentiation to mature sperm cells is believed to take place during the journey from the seminiferous tubule to the epididymis in the *in vivo* environment. Little is known, however, about the factors and conditions that regulate the differentiation of germ cells throughout the spermatogenesis process.

exerted directly on the germ cells or indirectly through their effects on the somatic cells is not clear.

Although it is known that somatic cells, extracellular matrix molecules, hormones and steroids influence the differentiation of germ cells, the exact nature of these interactions and of the molecules involved are not known. It appears that spermatogenesis results from the control and regulation of complex and diverse cellular interactions and communications.

An understanding of the ability of germ cells to switch from mitotic proliferation to terminal differentiation would be helpful for the understanding and treatment of cancer since tumors are known for their continued cell proliferation. In addition, the study of germ cells _in vitro_ would enable the study, diagnosis and treatment of infertility, and would be useful in evaluating potential gene therapy strategies to correct genetic defects.

Germ cells that have differentiated to mature sperm under controlled conditions would be useful for _in vitro_ fertilization or to produce transgenic animals, such as transgenic mice, by _in vitro_ fertilization.

Thus, a need exists for both immortalized or permanent cell lines, and conditionally immortalized cell lines capable of switching between proliferation and differentiation under appropriate conditions. Such cell lines can be used as _in vitro_ models of the somatic and germ cell lines to study spermatogenesis and for _in vitro_ fertilization as well as other purposes. Attempts at long term cultures of primary somatic cells and mixed primary somatic and germ cells have been largely unsuccessful. Such attempts have generally involved the _in vitro_ cultures of mixed cell types that seldom

In a further aspect, the present invention relates to immortalized somatic cell lines derived from mammalian testis capable of being cultured *in vitro* for at least one month or longer. Such somatic cell lines include Sertoli, myeloid peritubular and Leydig cell lines.

The immortalized somatic cell lines can be combined with the immortalized germ cell line to produce seminiferous tubule-like structures *in vitro*. The structures are useful as *in vitro* models for the study of spermatogenesis and cell-cell and cell-matrix interaction phenomena and to identify molecules involved in the process of tissue morphogenesis. The structures can be detected by labeling at least one cell line with a detectable marker, such as a fluorescent marker.

In another aspect of the present invention, the immortalized germ cell lines can be used to produce transgenic animals, such as transgenic mice.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an immunocytochemical analysis of germ cell lines GC-2spd(ts) and GC-3spd(ts) grown at 37°C (Figure 1A), and grown at 32°C (Figure 1B).

Figure 2A shows periodic acid-Schiff staining of the acrosomal granule in GC-2spd(ts) round spermatids; Figure 2B shows immunocytochemical staining of GC-2spd(ts) of the acrosomal granule in GC-2spd(ts) round spermatids with monoclonal antibody HS-63. Figure 2C shows an electron micrograph of the acrosomal granule.

Figure 3 shows flow cytometric analysis of GC-2spd(ts) cell line grown at 37°C for 16 generations (Figure 3A), 24 generations (Figure 3B), 30 generations

line in which the immortalizing molecule can be activated or inactivated under appropriate conditions. When the immortalizing molecule is activated, the cell line proceeds with mitotic proliferation, and when the immortalizing molecule in inactivated, the cell line proceeds to differentiate. A conditionally immortalized cell line can be constructed in a number of ways. A foreign gene encoding a temperature-sensitive immortalizing molecule, such as the polyoma large T antigen, can be integrated in the genome of a cell. The resulting cell line can be grown at temperatures at which the immortalizing molecule is active, or at which the molecule becomes inactive, such as between about 38°C and 40°C. In another embodiment, an immortalizing molecule can be operably linked to an inducible promoter that drives the expression of the immortalizing molecule. Alteratively, a cell line can be cotransformed with a gene encoding an immortalizing molecule and a gene encoding a molecule which binds with and inactivates the immortalizing molecule under certain conditions. For example, a cell line can be transformed with an immortalizing large T antigen, and a temperature-sensitive antiproliferative protein such as p53 capable of inactivating the large T antigen in the cell. As used herein, the term "conditionally immortalized germ cell line" refers to a germ cell line which has been conditionally immortalized but which is non-tumorigenic and non-malignant.

As used herein the term "antiproliferative protein" refers to tumor suppressor gene products such as cellular phosphoprotein p53 or Rb which have been found to complex with the transforming proteins of DNA tumor viruses in transformed cells. The p53 protein has been identified as associated with the large T antigen in SV-40 transformed cells (Lane et al. <u>Nature (London) 278</u>: 261-263 (1979), and Linzer et al., <u>Cell 17</u>:43-52 (1979)),

meiosis, while forcing the cells to cycle once again by mitosis. Other large T antigens useful in the present invention include, for example, polyoma virus large T antigens and temperature-sensitive polyoma large T antigens, as described in Linder et al., Exp. Cell Res. 191:1-7 (1990) and J. Chou, PNAS USA 75:1403-1413 (1978).

The methods for cultivating germ cell lines for the purpose of immortalizing them differ from previous attempts at the long term culture of non-tumorigenic germ cells is that the germ cells were grown in the presence of a monolayer of the somatic testicular cells prior to immortalizing with the large T antigen. Thus, the germ cells were cultured for a few days in a seminiferous tubule-like environment prior to immortalization.

Germ cells can be immortalized at defined stages of differentiation including, for example, primitive type A, type A or B spermatogonia, primary spermatocytes or secondary spermatocytes. Germ cells can be pre-selected at a desired differentiation stage before an immortalizing plasmid is incorporated into the genome. For example, germ cells taken from a testis corresponding to the different stages of differentiation can be separated using unit gravity sedimentation procedures, which are well known in the art. For example, the STA-PUT™ sedimentation procedure (Johns Scientific, Toronto, Canada) can be used, which is described in Romrell et al., Dev. Biol. 49:119-131 (1976). The type of immortalized germ cell line to be obtained therefore depends on the amount of differentiation a germ cell has undergone at the time the cell is extracted from a male animal.

An immortalized or conditionally immortalized germ cell line can be characterized according to its stage of differentiation by detailed microscopic and

proliferate. At a non-permissive temperature, the immortalizing molecule is inactivated, and the cells can proceed through differentiation. In another embodiment, an immortalizing molecule is operably linked to an inducible promoter that drives the expression of the immortalizing molecule.

Alternatively, activation and deactivation is controlled by a gene encoding a molecule which binds to and inactivates the immortalizing molecule inside the cell under certain conditions. Such cell lines are constructed by cotransfecting both a gene encoding for an immortalizing molecule and a gene encoding for an antiproliferative molecule capable of binding the immortalizing molecule under certain conditions. For example, primary germ cell populations enriched in secondary spermatocytes were cotransfected with a gene encoding an immortalizing molecule and a gene encoding an antiproliferative molecules capable of binding the immortalizing molecule. In one preferred embodiment, primary mouse germ cells were cotransfected with the SV40 Large T (LTAg) antigen gene and the gene encoding for a temperature-sensitive (ts) mutant of p53, to obtain cell lines in which both moelcules were expresssed. Two secondary spermatocyte cell lines, GC-2spd(ts) and GC-3spd(ts) were established in which an excess of p53 was able to bind LTAg at the permissive temperature, thus reducing the proliferative effects of LTAg. One of these cell lines in particular, GC-2spd(ts), was shown to be capable of differentiating and undergoing meoisis in vitro. Differentiation was characterized by immunocytochemical and morphological observation, as described in detail in Example V.

In another aspect, the present invention relates to methods of controlling the proliferation or differentiation of a conditionally immortalized germ cell

invention display normal morphology and are considered non-tumorigenic.

The present cell lines have been immortalized or conditionally immortalized from cells of the same developmental stage using a consistent and minimal genetic change, that is, introduction and stable integration of the pSV3-neo plasmid, and expression of the SV40 large T antigen gene, and for some cell lines the p53 gene as well. Previous attempts at long term cultures, on the other hand, have involved combining at least two cell types of the somatic cell environment or co-cultures of Sertoli and germ cells as the primary cultures.

Furthermore, the viability of these known primary co-cultures seldom exceeds fifteen days, whereas the immortalized somatic cell lines of the present invention have been cultured for more than 2 years. The known long-term cultures of such somatic cells have been derived generally from tumor cells compared with the immortalized non-tumorigenic cell lines of the present invention.

When cells from each of the immortalized somatic and germ cell lines are plated together, they reaggregate to form in vitro seminiferous tubule-like structures. The germ cells appear in the center of these formations, while Sertoli, peritubular and Leydig cells establish borders between these germ cell cores, similar to the structures produced by non-tumorigenic, freshly isolated testicular cells. Thus, immortalized germ cells can be cultured in the presence of fresh or immortalized somatic cells within the in vitro seminiferous tubule-like environment. Conversely, immortalized somatic cells can be used to grow and induce the differentiation of non-immortalized germ cells.

The following examples are intended to illustrate but not limit the following invention.

EXAMPLE I

Immortalization of Cells From Immature Mouse Testis

Testes from four 10-day old Balb/C mice were collected aseptically in serum-free CMRL-1066 culture medium (Gibco, Bethesda, MD), rinsed in 0.1 M phosphate buffered saline (PBS) and treated with collagenase (1 mg/ml in PBS) (Boehinger-Mannheim) for 15 minutes at room temperature. The medium was replaced by PBS containing DNase (10 ug/ml)(Sigma Chemical Co., St. Louis, MO) and the mixture pipetted up and down several times until a cell suspension was obtained. Cells were washed in PBS, then layered onto a discontinuous Percoll (Pharmacia, Piscataway, NJ) gradient composed of 4 layers with densities of 1.055, 1.045, 1.035 and 1.025, respectively, as described in Schumacher et al., FEBS LETT. 91:333-38 (1978). Centrifugation was carried out at 200 x g for 20 minutes in a bench-top centrifuge. Cells were distributed into 3 bands, corresponding to the 3 interphases of the Percoll gradient. Based on their morphology, adherence potential and growth characteristics, band A (density = 1.030) was enriched in spermatogonia and primary spermatocytes, band B (density = 1.040) in Sertoli and peritubular cells, and band C (density = 1.050) was enriched in Leydig and endothelial cells, both obtained from the same testes. Cells of each band were cultivated separately in tissue culture flasks (Falcon). Cell survival was optimal (10 days for spermatogonia, more than 20 days for somatic cells) in CMRL-1066 medium enriched with 80 μg/ml insulin, 3 μg/ml transferrin, 80 μg/ml ascorbic acid and 13% inactivated fetal calf serum.

EXAMPLE II

Integration of the SV40 Large T Antigen Gene

After trypsinization, cells were washed once in PBS and pelleted by centrifugation. Genomic DNA was recovered from cell pellets by treatment with 1% SDS and 50 μg/ml proteinase K in TE buffer (0.01 Tris, 0.001 M EDTA, pH 7.5). DNA was extracted with phenol and chloroform and recovered by ethanol precipitation according to well known standard methods as described in Sambrook et al., Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory Press (N.Y. 1989), incorporated herein by reference. Fifteen μg of genomic DNA and 1 ng of pSV3-neo DNA (positive control) were digested with Bam HI in order to excise the large T antigen gene. The samples containing PG3 peritubular cell DNA, SH2 Sertoli cell DNA, LAB2 Leydig cell DNA and germ cell DNA were electrophoresed in 0.8% agarose gel and transferred onto a nitrocellulose filter. The filter was hybridized with a ^{32}P-labelled Bam HI fragment of the pSV3-neo.

All the permanent cell lines integrated the SV40 large T antigen gene in their genome as shown by conventional Southern blot analysis of the cellular high molecular weight DNA. A 3.3 kb fragment was detected that corresponds to the large T antigen gene. These results confirm that the immortalizing gene has been integrated in the genome of these cells. In addition, these cells show expression of the large T antigen with the typical nuclear localization, as shown by immunocytochemistry.

A. Test for Malignant Transformation

The use of oncogenes to immortalize cells is known to result occasionally in the malignant transformation of the cells as evidenced by their ability to grow in soft agar and/or in nude mice. To determine whether the immortalized cells had undergone malignant transformation, cell monolayers were first trypsinized, treated with 1 mg/ml collagenase in PBS until a single-cell suspension was obtained and washed several times in PBS. Soft agar cultures were performed following the method of Hamburger and Salmon, Science 197:461 (1977), incorporated herein by reference. Briefly, $1x10^6$ cells were resuspended in warm CMRL-1066 medium fortified as described in Example I and containing 0.3% agar noble (Difco Laboratories, Detroit, MN). The mixture was poured onto an underlayer previously prepared in 60 mm diameter culture dishes. The underlayer was made of 0.6% agar in McCoy's medium 5a completed with 1% sodium pyruvate, 42 μg/ml L-serine, 1% glutamine (200 mM), 0.15% tryptic soy broth, 1% penicillin/strep and 13% inactivated fetal calf serum. Cultures were incubated at 37°C and 5% CO_2 for at least one week before assessment of clonal proliferation. Only between 12.5% and 32.5% of the immortalized clones were able to grow in soft agar, i.e., 1 out of 8 Sertoli (SC72), 3 out of 22 Leydig (LFA2, LED2 and LAH7) and 5 out of 16 peritubular (PG52, PB51, PF71, PE102 and PB83) cells, while the majority of the immortalized somatic cell lines and our immortalized germ cell clone (GC-1) had retained a normal growth behavior including contact inhibition. Contact inhibition is a property of non-tumorigenic, non-malignant cells in which the cells proliferate until they come into contact with adjacent cells to establish a monolayer. Malignant cells, on the other hand, will not

C. Immunohistochemical Characterization

To characterize the specific markers expressed by the different immortalized cell types, the immortalized cells were cultured to confluency in Lab-Tek chamber slides (Nunc, Naperville, IL) with complete D-MEM medium and fixed with cold methanol. Antigens were revealed by using an immunoperoxidase technique according to the manufacturer's directions (Vectastain ABC kit, Vector, Burlingame, CA). The primary antibodies used were mouse monoclonal anti-SV40 large T antigen (1:100 in PBS) (Oncogene Science, Manhasset, NY), and mouse monoclonal anti-desmin (1:100 in PBS) (Amersham, Arlington Heights, IL). The rabbit polyclonal and mouse monoclonal antibodies against the testis-specific LDH-C isozyme and the rabbit polyclonal antibodies against the testis-specific isoform of cytochrome c (designated cytochrome c_t) were supplied by Dr. Erwin Goldberg (Northwestern University, Illinois). Counterstaining was performed with hematoxylin, fast green or neutral red.

Two histochemical markers were detected. The 3ß-hydroxysteroid dehydrogenase (3ß-HDH) assay, an enzyme that converts pregnenolone to progesterone and is characteristic of Leydig cells, was performed according to the method described by Wiebe et al. in Endocrinology 98:505-513 (1976), using dehydroepiandrosterone, ß-NAD and nitro blue tetrazolium. After the reaction was completed, cells were fixed in 4% formalin in PBS, pH 7.2 (buffered formalin) and counterstained with 0.1% neutral red. For detection of total alkaline phosphatase activity, Leydig and peritubular cells were first fixed with buffered formalin, stained with Naphthol As-MX phosphate (Sigma Chemical Company, St. Louis MO) and Fast Violet B salt (Sigma Chemical Co.) by a well known procedure described in Wiebe, Endocrinology 98:505-513 (1976), which is incorporated herein by reference, and

dehydrogenase (LDH-C). These isozymes are recognized as being specific for the spermatogenic lineage and expressed from the preleptotene stage of germ cell differentiation onward. The GC-1 line is a permanent cell line capable of expressing isoproteins in culture. No other permanent cell line is known to express these isoproteins in vitro. The immunohistochemical staining of the GC-1 cell line using a monoclonal antibody against LDH-C and a monospecific rabbit polyclonal antibody to the testicular isoform of cytochrome c demonstrated the reactivity of the antibody to LDH-C and cytochrome c. The antibodies against LDH-C and cytochrome c_t are described and identified in Goldberg et al., Science 196:1010-1012 (1977), incorporated herein by reference.

The designation of primary spermatocyte usually implies that the cell in question has stopped dividing by mitosis, has differentiated and is entering the 1st meiotic division. Both the LDH-C and cytochrome c_t gene products are known to start being expressed at the preleptotene/leptotene transition. By immortalizing the germ cell line at this stage, the large T antigen expression (a nuclear factor that normally regulates SV40 viral replication) has "locked" the cells at the developmental, and gene expression, stage of early meiosis while forcing the cells to cycle again by mitosis resulting in the proliferation of the cells and continued expression of the isoproteins.

EXAMPLE IV

Formation of In Vitro Tubule-like Structures

One characteristic of the in vitro immortalized cell lines of the present invention is their ability to associate and reconstitute seminiferous tubule-like structures when plated together. The primary spermatocyte line GC-1 appears in the center of these

A. Obtaining Cell Lines

A single cell suspension was obtained from decapsulated testes of sexually mature Balb/c mice as previously described above in Example I above. A cell fraction enriched with spermatocytes was then isolated using the STA-PUT™ (Johns Scientific, Toronto, Canada) gravity sedimentation procedure at 4°C according to the procedure described in Romrell et al, Dev. Biol. 49: 119-131 (1976), and Peden et al, Virol 168, 13 (1989), each incorporated herein by reference. Spermatocytes were cultivated in 60 mm tissue culture dishes (Falcon) and completed CMRL-1066 medium until a monolayer was formed.

Cotransfection was performed with two plasmids, the pSV3-neo plasmid which contains the LTAg gene and the neo^r gene, and the LTRp53cG9 plasmid which contains the temperature sensitive $[val^{135}]$p53gene, and which was previously described in Michalowitz et al. Cell 62, 671-680 (1990) and Martinez et al. Genes & Develop. 5, 151 (1990), each incorporated herein by reference, and was provided by Dr. Channing Der, University of North Carolina, Chapel Hill, North Carolina. Cotransfection of 12.5μg pSV3-neo (ATCC #37150) and 12.5μg LTRp53cG9 per dish was performed by the calcium phosphate method described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), incorporated herein by reference. G418 (Geneticin, Gibco, Chagrin Falls, OH), at a concentration of 200 μg/ml of active substance, was used for the selection of neomycin resistant colonies.

After selection with G418, two cell clones, designated GC-2spd(ts) and GC-3spd(ts) were obtained and cultivated after crisis for a period of six months.

cells, LDH-C and cytochrome c_t, were expressed at low levels. p53 overexpression was confined to the cytoplasm.

37°C: At 37°C, both cell lines grew more slowly, with a doubling time of 24 hours. Figure 1 shows staining patterns for p53, LDH-C, and cytochrome c_t for GC-2spd(ts) and GC-3spd(ts) at 37°C (Figure 1A) and 32°C (Figure 1B). At 37°C, LDH-C expression was enhanced, but cytochrome c_t was not identifiable by immunocytochemistry, as shown in Figure 1A. The p53 protein was expressed in both the cytoplasm and the nucleus in both cell lines.

At 37°C, groups of cells showed signs of morphologic differentiation already visible at the light microscopy level. In some cells of both lines, but particularly in GC-2spd(ts), a dark granule appears at one pole of the nucleus, whereas the cell cytoplasm at the other pole of the nucleus become elongated.

Morphological differentiation of GC-2spd(ts) was examined by growing the cell lines to confluency in a LabTek chamber and fixed with cold methanol. Periodic acid-Schiff (PAS) staining was performed according to the methods described in Sheenan et al. Theory and Practice of Histotechnology. Battelle Press, Columbus, OH (1987). Immunocytochemical staining was performed as described above (Example III), using a monoclonal antibody, HS-63 (donated by Erv Goldberg, Northwestern University, Evanston, IL), against the sperm acrosome antigen MSA-63 (as described in Liu et al, Biol Repr 46, 937 to 948 (1992). For electronmicroscopy, cell monolayers were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, postfixed in OsO_4 and embedded in an epoxy resin by usual methods. Ultrathin sections were stained with uranylacetate and lead citrate, and visualized with a JEOL 100 cx electron microscope.

same cells, which show a diploid cell cycle, as well as an extra haploid peak. Figure 3C shows the 30th generation of the same cells, which shows an increase of the percentage of haploid cells. Figure 3D shows control mouse testis cells.

Haploidy was shown only at 37°C, indicating that the cell cycle has to be maintained in order for the cells to complete the second meiotic division. It appeared that certain cells belonging to a pool of continually dividing secondary spermatocytes were able to proceed through the second meiotic division and to differentiate into spermatids. This phenomenon was attributed to the behavior of the p53 protein at 37°C in this cell line. The borderline temperature of 37° seemed to allow the expression of both mutated and wt p53.

In contrast, the GC-3spd(ts) cell line did not progress into meiosis. In this line, p53 was expressed almost exclusively in the cytoplasm (mutant form) at 37°C, and only in the nucleus (wt form) at 32°C. Since at this temperature the cell cycle is blocked, the cells were probably prevented from undergoing the second meiotic division.

<u>32°C</u>

At 32°C, in both lines, cell growth was slowed progressively and the cells died after an average of 10 generations. This was attributed to the expression of the wild-type exogenous p53 gene that, in excess, blocked the immortalizating action of LTAg and arrested the cell cycle. In these cells, p53 was expressed almost exclusively in the nucleus. LDH-C and cytochrome ct expression were markedly enhanced as is seen in Figure 1. The germ cell line bearing only the LTAg, GC-1, continued to proliferate at 32°C and maintained the same morphological features, whereas GC-2spd(ts) and GC-

We claim:

1. An immortalized germ cell line.

2. The immortalized germ cell line of claim 1, wherein said germ cell line is of murine origin.

3. The immortalized germ cell line of claim 1, wherein said germ cell line is a spermatogonia, primary spermatocyte, secondary spermatocyte or spermatid.

4. The immortalized germ cell line of claim 3, wherein said germ cell line expresses a testicular isoform of cytochrome c and LDH-C.

5. The immortalized germ cell line of claim 1, wherein said germ cell line is GC-1.

6. A method for producing an immortalized non-tumorigenic germ cell line comprising:

(a) growing a non-tumorigenic germ cell in the presence of non-tumorigenic Sertoli, peritubular and Leydig cells; and

(b) immortalizing said germ cell with an immortalizing molecule.

7. The method of claim 6, wherein said immortalizing molecule is a large T antigen.

8. The method of claim 7, wherein said large T antigen is an SV40 large T antigen.

9. The method of claim 6, wherein said immortalizing molecule is transfected into the genome of said germ cell to immortalize said germ cell.

17. A conditionally immortalized germ cell line capable of undergoing meiosis and differentiation *in vitro*.

18. The conditionally immortalized germ cell line of claim 16 wherein the germ cell line is a murine testicular germ cell line.

19. The conditionally immortalized testicular germ cell line of claim 17 wherein the germ cell line is secondary spermatocyte.

20. The conditionally immortalized germ cell line of claim 17 wherein the germ cell line comprises a first foreign gene encoding an immortalizing molecule and a second foreign gene encoding a temperature sensitive molecule which inactivates the immortalizing molecule at a temperature of 37°C or less.

21. The conditionally immortalized germ cell line of claim 19 wherein the first foreign gene encodes the large T antigen.

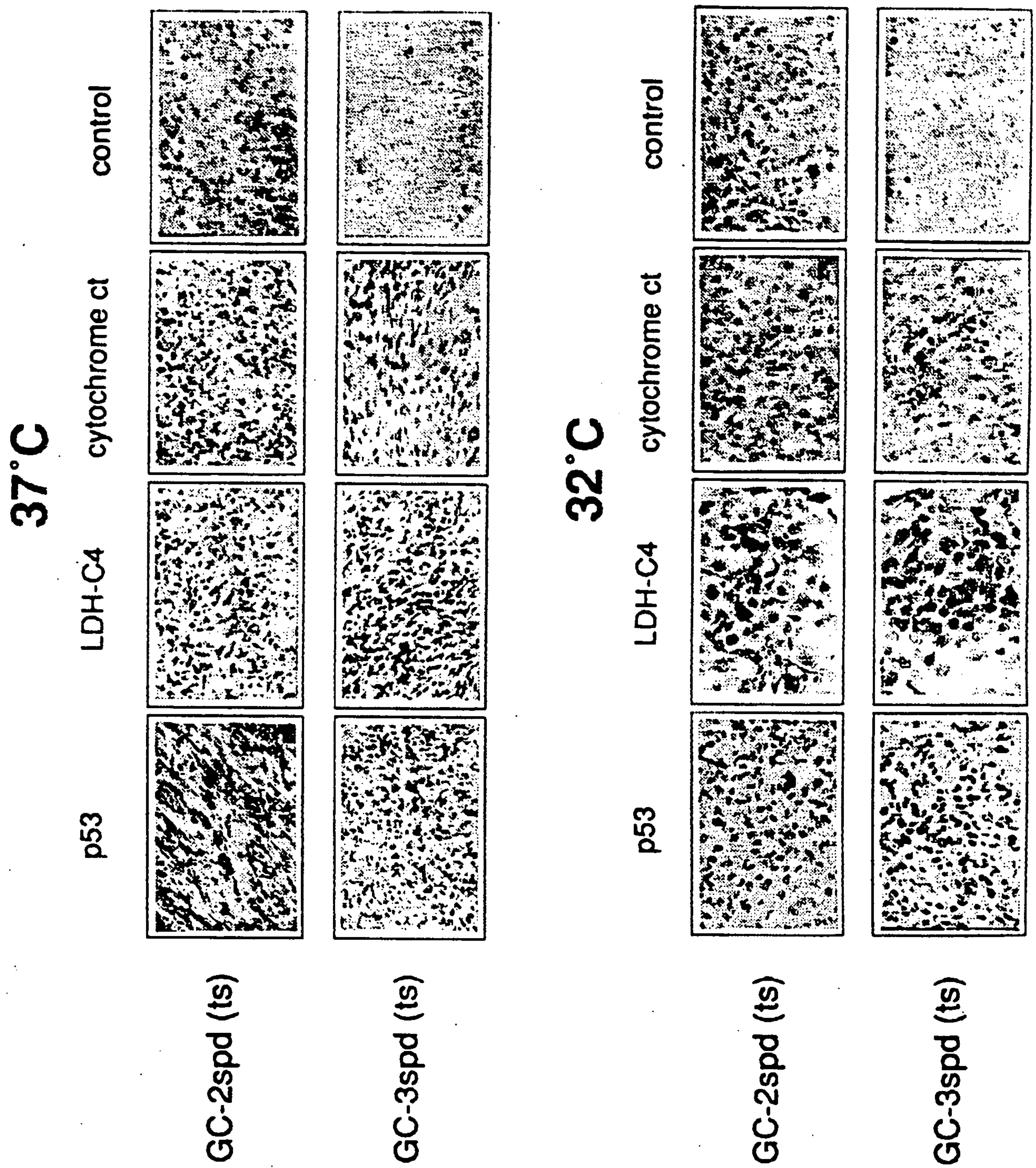
22. The conditionally immortalized germ cell line of claim 19 wherein the second foreign gene encodes a molecule which binds to the immortalizing molecule is an antiproliferative protein capable of binding to the immortalizing molecule inside the cell.

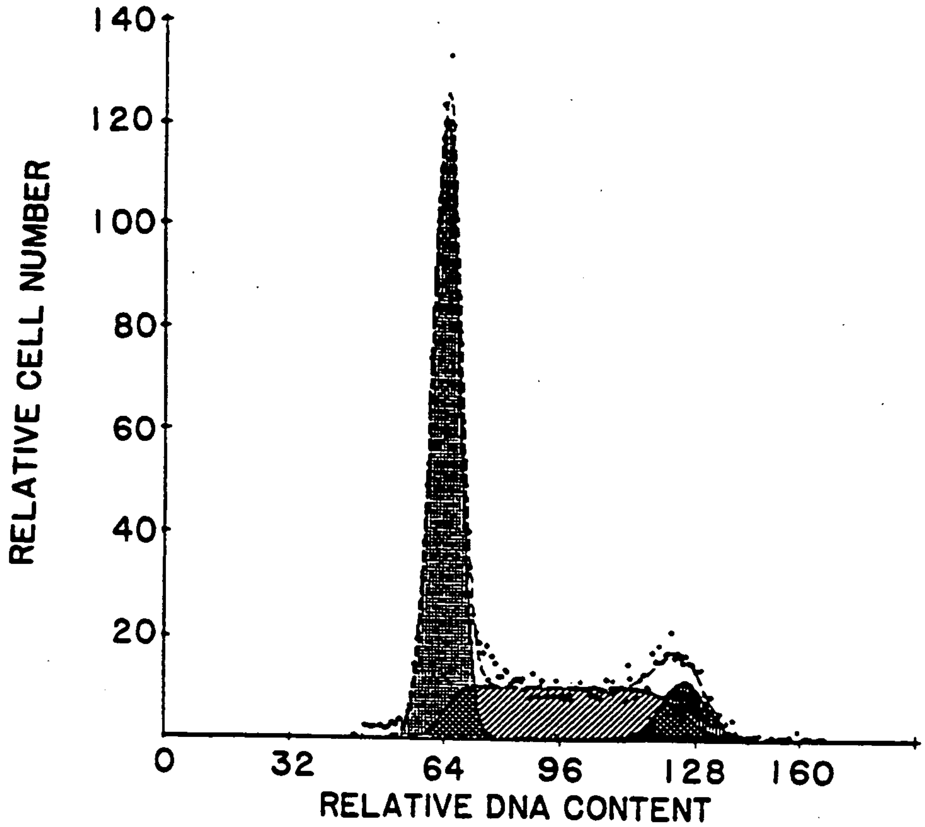
23. The conditionally immortalized germ cell line of claim 21 wherein the antiproliferative protein is p53.

24. The conditionally immortalized germ cell line of claim 22 wherein the cell line is GC-2spd(ts) or GC-3spd(ts).

FIG. IA

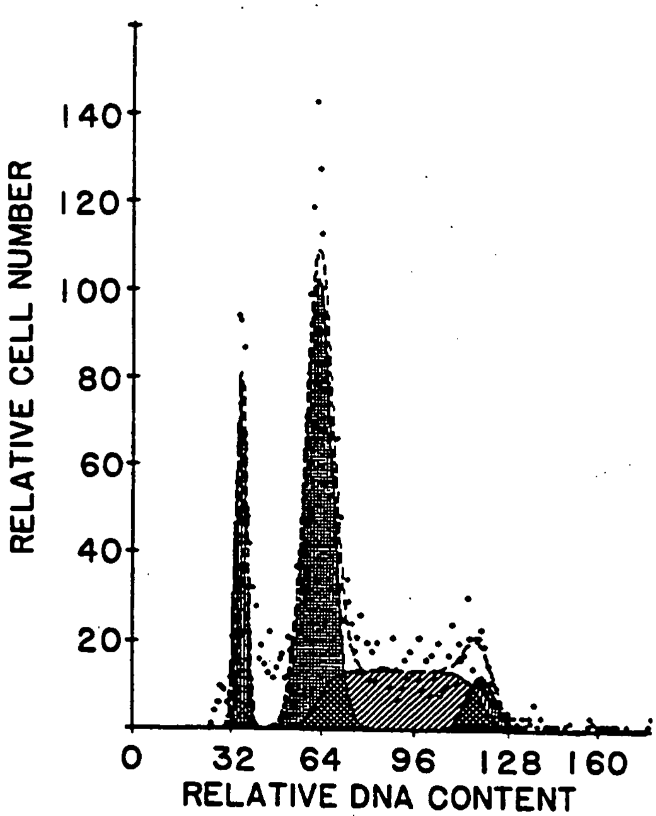
FIG. IB


37°C
p53
LDH-C4
cytochrome ct
control
GC-2spd (ts)
GC-3spd (ts)
32°C
p53
LDH-C4
cytochrome ct
control
GC-2spd (ts)
GC-3spd (ts)



RELATIVE CELL NUMBER
140
120
100
80
60
40
20
0
32
64
96
128
160
RELATIVE DNA CONTENT

FIG. 3A



RELATIVE CELL NUMBER
140
120
100
80
60
40
20
0
32
64
96
128
160
RELATIVE DNA CONTENT

FIG. 3B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07654

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/10, 15/87
US CL :435/240.2, 172.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240. 172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE EXPRESS, BIOSIS, WPI
search terms: testicular, testes, germ ceil

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Biology of Reproduction, Volume 23, issued 1980, J. P. Mather, "Establishment and Characterization of Two Distinct Mouse Testicular Epithelial Cell Lines", pages 243-252, entire document.	1-4 ---- 5-31
X --- Y	Annals of the New York Academy of Sciences, Volume 383, issued 1982, J. P. Mather et al., "CULTURE OF TESTICULAR CELLS IN HORMONE-SUPPLEMENTED SERUM-FREE MEDIUM", pages 44-68, entire document.	1-4 ---- 5-31
X --- Y	Journal of Ultrastructure Research, Volume 87, issued 1984, J. P. Mather et al., "Establishment of a Peritubular Myoid-like Cell Line and Interactions between Established Testicular Cell Lines in Culture", pages 263-274, entire document.	1-4 ---- 5-31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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